

Sperm counts in enzymatically liquefied cervical mucus: quantitative validation using donor cervical mucus

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The post-coital test evaluates the penetration of spermatozoa into cervical mucus; it relies on subjective measurements and therefore lacks precision. Enzymatic liquefaction of cervical mucus allows sperm concentration to be measured in post-coital test samples, but the reliability of such measurements is not known. Donor cervical mucus was used as a model to test the accuracy and sensitivity of sperm quantification in liquefied cervical mucus. Donor cervical mucus was dissolved by enzymatic treatments in the presence of known numbers of spermatozoa and the recovery of sperm cells was assessed after liquefaction of the samples. Enzymatic treatment of cervical mucus with a combination of bromelain and glycosidases resulted in reliable and fast liquefaction of the samples. The accuracy of sperm concentration measurements was $89 \pm 10\%$ (mean \pm SD, $n = 50$), and the sensitivity limits were 1×10^6 and 0.2×10^6 spermatozoa/ml for quantitative concentration measurement and qualitative sperm detection respectively. This study demonstrates that liquefaction of cervical mucus by combined protease and glycosidases allows accurate and sensitive determination of sperm concentration in the sample. Therefore we believe that valuable data can be obtained for sperm concentration and total sperm counts in post-coital tests, that should help to improve the reliability of the post-coital test.

Key words: cervical mucus/enzymatic liquefaction/infertility/post-coital test/spermatozoa

Introduction

The evaluation of the interactions between sperm cells and cervical mucus is of clinical relevance but suffers from methodological drawbacks, rendering it highly subjective (Griffith and Grimes, 1990). The parameter most affected is sperm concentration, which, when assessed in the visco-elastic milieu of cervical mucus, remains of unknown accuracy due to variations in the thickness of the sample examined. In addition, spermatozoa are not homogeneously distributed in cervical mucus but are known to migrate in a columnar mode (Kremer and Jager, 1988).

Cervical mucus liquefaction using the cysteine protease bromelain has been used to detect antisperm antibodies and

leukocytes in the mucus as well as to quantify sperm in the post-coital test (PCT) (Ingerslev and Poulsen, 1980; Thompson *et al.*, 1991; Campana *et al.*, 1991; Shulman and Hu, 1992). Enzymatic liquefaction of cervical mucus reduces its viscosity and allows the sample to be mixed so that sperm cells become evenly distributed. Therefore it is possible to determine the concentration (and hence the total number) of sperm cells present in liquefied cervical mucus from PCT using conventional Makler chambers. Such quantification could greatly improve the quality of data obtained from PCT and enable normal values for sperm colonization of cervical mucus to be derived. However, the accuracy and sensitivity of sperm concentration measurements cannot be determined in liquefied PCT samples, due to the fact that the actual number of spermatozoa present is unknown.

Therefore, the present study has been designed to validate the determination of sperm concentration in liquefied cervical mucus. Instead of PCT samples, we have used as a model donor cervical mucus admixed with known numbers of sperm cells prior to enzymatic liquefaction. Thus the accuracy and the sensitivity of sperm concentration determinations in liquefied cervical mucus could be evaluated, and the effects of enzyme treatments on sperm counts tested. In addition, we have compared the efficiency of bromelain and of a mixture of bromelain and glycosidases to liquefy cervical mucus. The data presented demonstrate that accurate and sensitive measurements of sperm concentration can be achieved in liquefied cervical mucus, and thus certify that sperm concentration can be reliably measured in PCT.

Materials and methods

Materials

Bromelain (EC 3.4.22.4) and hyaluronidase (EC 3.2.1.36) were purchased from Sigma (St Louis, MO, USA). α -Amylase was obtained from Merck (Darmstadt, Germany). The enzyme cocktail was prepared from 3-fold concentrated stock solutions by mixing equal volumes of the three enzymes at the time of the experiment. Enzyme concentrations in the cocktail were bromelain (6 mg/ml), α -amylase (4.5 mg/ml) and hyaluronidase (9 IU/ml). For experiments one volume of cocktail was admixed with two volumes of sample (either spermatozoa and cervical mucus or spermatozoa and buffer) to yield final enzyme concentrations of 2 mg/ml, 1.5 mg/ml and 3 U/ml, respectively. All enzymatic digestions were performed in modified Biggers–Whitten–Whittingham (BWW) buffer without albumin (Biggers *et al.*, 1971).

For determination of sperm concentrations, spermatozoa were counted in Makler chambers (Sefi Medical Instruments, Haifa, Israel) according to the manufacturer's instructions. Determinations were made in duplicate using the whole grid of the cell (volume = 0.01 μ l).

Spermatozoa

Spermatozoa were obtained from liquefied ejaculates used for routine sperm analysis in the andrology laboratory. The sperm cells were diluted in BWB buffer to the appropriate concentration.

Donor cervical mucus

Midcycle donor cervical mucus was obtained at the time of oocyte retrieval from patients undergoing in-vitro fertilization (IVF) and treated with exogenous gonadotrophins as previously published (Sakkas *et al.*, 1994). Cervical mucus was collected in an Aspiglaire device (Biotechnologie International, l'Aigle, France) consisting of a plastic transparent capillary with an inner piston. The external os of the cervix was then wiped with a dry sterile cotton swab. While the piston of the Aspiglaire was completely pushed forward, the tip of the Aspiglaire was then introduced through the external os and advanced through the cervical canal very slowly and gently until the tip reached the level of the internal cervical os. At this point slow aspiration of the cervical mucus started while slowly and carefully retracting the Aspiglaire. When the procedure was completed, the Aspiglaire was immediately handed to the andrology laboratory. This method is similar to that described in the World Health Organization (WHO) guidelines manual, using a tuberculin syringe without needle (WHO, 1992).

Cervical mucus score

Macroscopic parameters of cervical mucus score (pH, consistency, spinnbarkeit and ferning) were measured on the endocervical part of the mucus, i.e. the part close to the piston of the Aspiglaire, at the time of extrusion of the sample into 3 ml polycarbonate tubes. The criteria or scoring were as described in the WHO guidelines (WHO, 1987, 1992). In all cases the score of donor cervical mucus was >10.

Liquefaction of donor cervical mucus in the presence of sperm cells

Aliquots of donor cervical mucus (200 µl) were admixed in a 3 ml polycarbonate tube with 200 µl of sperm suspension containing $3\text{--}6 \times 10^6$ sperm cells and mixed on a vortex mixer. At time $t = 0$, 200 µl of enzyme cocktail was added, the tube was thoroughly mixed and incubated at 37°C in a thermostatically heated block. Liquefaction was assessed by gently tilting the tube on its side every 10 min. When liquefaction was complete as judged by the sample behaving as a single fluid non-viscous phase, two aliquots of 7 µl were withdrawn and placed in Makler chambers for immediate counting. The remaining samples were further incubated to a total time of 90 min, and then two more samples were counted.

Incubation of sperm cells with enzymes: control experiments

In parallel with donor cervical mucus liquefaction, triplicate tubes containing sperm cells from the same donor were incubated in the same conditions (dilution and enzyme treatment), but with buffer substituting for cervical mucus. The samples were counted in duplicate in a Makler chamber at times $t = 0$, $t = 60$ min and $t = 90$ min. Means and SD of concentrations were calculated from the triplicate samples at each time point. The value obtained at time $t = 0$ was used as 100% for converting counts obtained at subsequent time points to percentage as well as for comparison with sperm counts in liquefied donor cervical mucus. Unless otherwise stated in the text, the initial sperm concentration in different experiments was between 5×10^6 and 10×10^6 spermatozoa/ml and was kept constant between the control samples and the corresponding test samples containing cervical mucus. This concentration range was chosen because it is in the measuring range of Makler cells and it also corresponded to sperm concentrations found in PCT. The sperm concentration used in each experiment was determined by the amount of donor material

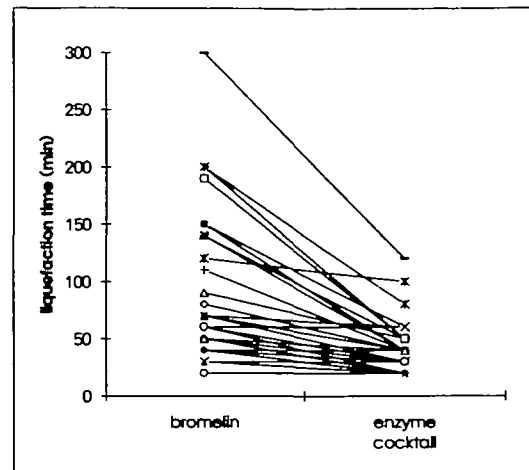


Figure 1. Cervical mucus liquefaction using bromelin or enzyme cocktail. Each donor cervical mucus was divided into two parts, one being incubated with bromelin (2 mg/ml) the other with the enzyme cocktail, and liquefaction times were measured ($n = 34$). Liquefaction times were found to be significantly shorter for the samples incubated with the enzyme cocktail as compared to those incubated with bromelin (Wilcoxon signed rank test, $P < 0.01$).

available. Thus, the absolute counts at $t = 0$, which were used as 100% in individual experiments, were contained within a 2-fold range, i.e. $5\text{--}10 \times 10^6$ spermatozoa/ml, and normalization of the sperm counts in percentages therefore allowed better comparison of the results of different experiments.

Statistical methods

Liquefaction times of donor cervical mucus treated in parallel with the enzyme cocktail and with bromelin were compared by the Wilcoxon signed rank test. The limit in sensitivity of sperm concentration measurement in liquefied cervical mucus was determined by Levene's test (comparison of SD) including or not including values $< 0.5 \times 10^6$ spermatozoa/ml. $P < 0.05$ was considered significant.

Results

Cervical mucus liquefaction time with different enzymes

To select reliable conditions to solubilize cervical mucus we have compared liquefaction times obtained for aliquots of the same donor cervical mucus incubated either with the combination of bromelin, α -amylase and hyaluronidase (hereafter designated as 'enzyme cocktail') or with bromelin alone (2 mg/ml). In a series of 34 different mucus samples, the enzyme cocktail produced liquefaction at a median time of 40 min, with individual values ranging between 20 and 120 min, while in the presence of bromelin alone the median liquefaction time was 60 min with a range 20–300 min (Figure 1). After 60 min of incubation 53% of the samples treated with bromelin and 91% of the samples treated with the enzyme cocktail were liquefied while after 90 min 71% of the samples treated with bromelin and 94% of the samples treated with the enzyme cocktail were liquefied. Statistical analysis showed that liquefaction times were significantly shorter in the group incubated with the enzyme cocktail (Wilcoxon signed rank test, $P < 0.01$). Therefore treatment of cervical mucus with

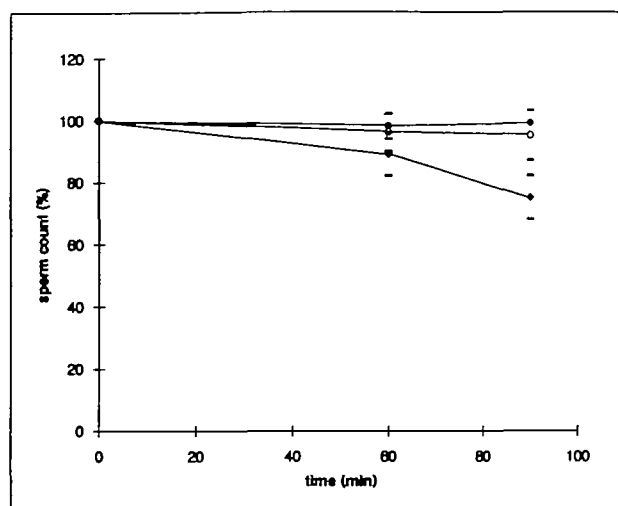


Figure 2. Effect of enzyme treatments on sperm counts. Sperm cells were incubated with control buffer (closed circles), bromelin 2 mg/ml (open circles) or enzyme cocktail (see text; closed diamonds), and counted at times $t = 0$, $t = 60$ min and $t = 90$ min. Counts are expressed as percentage of the value obtained at time $t = 0$, each point representing 11–28 different sperm samples (mean \pm SD). Bromelin did not affect sperm counts, and the enzyme cocktail induced 11 and 25% decreases in sperm recovery after 60 and 90 min incubation respectively.

the enzyme cocktail, rather than with bromelin alone, resulted in shorter and more reliable liquefaction times.

Cervical mucus liquefaction was originally proposed using a concentrated bromelin preparation diluted 1:1 with the sample to a final concentration of 5 mg/ml. Due to the poor solubility of bromelin commercial preparations in physiological buffers, we have reduced this final concentration to 2 mg/ml. We have verified that cervical mucus liquefaction times were not different when using bromelin at 2 rather than 5 mg/ml by incubating in parallel aliquots of the same donor cervical mucus ($n = 14$). No significant differences in liquefaction times were observed ($P > 0.05$, data not shown).

Effect of enzyme treatment on sperm counts: bromelin 2 mg/ml and cocktail

Data from Figure 1 suggested that the use of the enzyme cocktail may be beneficial for sperm concentration measurements in liquefied cervical mucus from PCT. We have compared the effects of treatment with the enzyme cocktail or with bromelin (2 mg/ml) on sperm counts by incubating sperm cells with enzymes in the absence of cervical mucus. Each experiment was performed with spermatozoa from a different individual and involved parallel incubations with the various enzymes and a control incubation in buffer alone, as described above. Sperm concentrations were measured after 60 and 90 min incubation, a time range that covered liquefaction times of most of the samples according to data presented in Figure 1. Figure 2 shows that, in the absence of enzymes, as well as in the presence of bromelin, sperm counts remained stable over a 90 min incubation period, whereas they gradually decreased when sperm cells were incubated with the enzyme cocktail. After 60 min of incubation with the enzyme cocktail, sperm counts, expressed as percentage of the value at $t = 0$,

were $89 \pm 7\%$ (mean \pm SD, $n = 24$) and further decreased to $75 \pm 7\%$ ($n = 22$) at 90 min. The narrow dispersion of values obtained, resulting in SD $< 10\%$, reflected the lack of individual variation in sperm sensitivity to the enzymes. The decrease in sperm counts observed with the enzyme cocktail was attributed to gradual sperm lysis by the enzymes and was attested by the presence of isolated sperm tails in the preparations (data not shown).

Sperm counts in cervical mucus treated with the enzyme cocktail: difference between counts at liquefaction time and after 90 min

The data outlined above suggested that when sperm cells were incubated with the enzyme cocktail, progressive cell lysis occurred, resulting in sperm counts decreasing with the incubation time. The presence of cervical mucus may minimize sperm lysis by providing alternative substrates for these enzymes, acting as competitive inhibitors. To investigate the effects of the enzyme cocktail on sperm counts during cervical mucus liquefaction, cervical mucus samples were incubated with the enzyme cocktail in the presence of sperm cells as described above. The sperm cells were counted at the time of mucus liquefaction, which varied for each sample, and after a total of 90 min incubation, a common time for all samples. Sperm counts were expressed as percentage of the counts in control samples at $t = 0$. When spermatozoa were counted at liquefaction time, the percentages of sperm cells recovered were similar for liquefaction times ranging from 20 to 120 min and the variation observed were within the range of experimental error ($88\% \pm 7\%$, mean \pm SD, $n = 35$) (data not shown). To analyse the effects of prolonged incubations with the enzyme cocktail we have calculated the difference in sperm recoveries between counts performed at liquefaction time and at 90 min. Figure 3 shows the difference between sperm counts obtained at 90 min and at liquefaction time plotted as a function of the mucus liquefaction time. Thus, a zero value on the y axis meant that no difference was observed between counts at liquefaction time and after 90 min, while negative values meant that sperm counts at 90 min were lower than sperm counts taken at liquefaction time.

The data presented in Figure 3 show that in most cases sperm counts obtained after 90 min of incubation with the enzyme cocktail were lower than sperm counts taken at liquefaction time. This was most evident in samples with short liquefaction times (20–40 min), in which sperm cells were exposed with enzymes for at least 50 min after the completion of liquefaction.

These results showed that, notwithstanding the variability in liquefaction time between different mucus samples, sperm counts obtained at liquefaction time were more accurate than those obtained after a fixed incubation time of 90 min. Thus, in order to avoid underestimated counts due to sperm lysis by the enzyme cocktail, it was considered that sperm concentrations should be determined within 30 min after mucus liquefaction, or better, at the time of liquefaction. This could be accomplished by testing the samples for liquefaction every 15 min and immediately counting the sperm cells when liquefaction is observed.

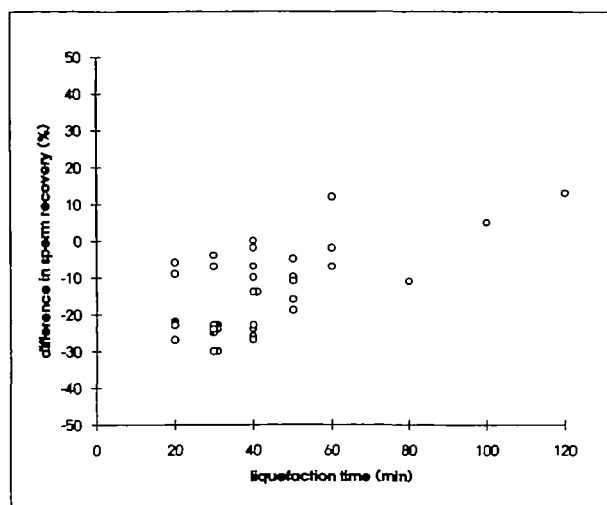


Figure 3. Effect of enzyme cocktail on sperm counts in presence of cervical mucus. Sperm cells were incubated with donor cervical mucus and enzyme cocktail and counted first at liquefaction time and again at time $t = 90$ min. Sperm counts were expressed as percentage of counts at time $t = 0$, obtained in control samples where cervical mucus was replaced by buffer. The difference between sperm counts at liquefaction time and at 90 min was plotted according to the mucus liquefaction time. Sperm counts ranged between 73 and 102% at liquefaction time and between 60 and 100% after 90 min incubation ($n = 35$).

Sperm concentration in liquefied cervical mucus: accuracy of the method

To estimate the reliability of determination of sperm concentration present in PCT samples, the accuracy of sperm concentration measurements in liquefied donor cervical mucus was assessed. To this end, known numbers of sperm cells were mixed with donor cervical mucus, the samples were liquefied with the enzyme cocktail, and sperm cells were counted. These experiments were performed using sperm concentrations of $5\text{--}10 \times 10^6$ spermatozoa/ml. Sperm counts were taken at liquefaction time and normalized to the counts of control samples at time $t = 0$. Control samples were sperm cells from the same stock suspension diluted with buffer instead of donor cervical mucus as described above. Figure 4 shows sperm counts in liquefied cervical mucus as a function of liquefaction time. These data indicate that, in 92% of the samples the sperm cells recovered from liquefied cervical mucus represented over 80% of the original number of spermatozoa added to the preparation, whereas in the remaining 8% of the samples the number of sperm cells recovered was between 73 and 80%. The average recovery obtained was $89 \pm 10\%$ (mean \pm SD, $n = 50$). These results demonstrated that liquefaction of cervical mucus by the enzyme cocktail allowed reliable and accurate determination of sperm concentration. The variability of 20% observed in individual sperm recoveries after cervical mucus liquefaction seemed acceptable given the complex nature of this biological system, and should not have significantly affected the sensitivity of sperm concentration measurements.

Less than 10% variation was obtained using liquefied semen (see Figure 2). In the case of cervical mucus liquefaction, adequate concentrations of spermatozoa ($5\text{--}10 \times 10^6$

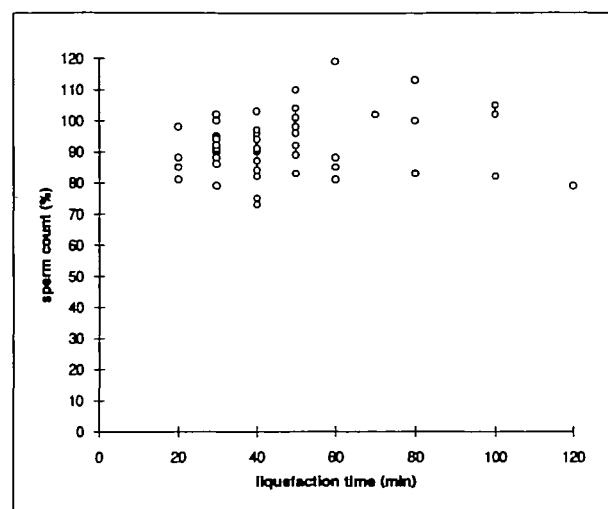


Figure 4. Accuracy of sperm counts in liquefied cervical mucus. Sperm cells were incubated with donor cervical mucus and enzyme cocktail, and counted at liquefaction time. Sperm counts were expressed as percentage of the counts at time $t = 0$, obtained in control samples where cervical mucus was replaced by buffer, and plotted as a function of liquefaction time. Average recovery of spermatozoa in 50 samples was $89 \pm 10\%$ (mean \pm SD).

spermatozoa/ml) were counted, and the wide variations observed may be related to the complexity of the biological system rather than to the use of the Makler counting chamber.

Sperm counts in liquefied cervical mucus: sensitivity of the method

Finally, the lowest sperm concentration allowing accurate measurements of sperm concentration in liquefied cervical mucus was determined. In addition, the lowest sperm concentration allowing reliable detection of sperm cells in liquefied cervical mucus was estimated. Decreasing concentrations of spermatozoa were incubated with donor cervical mucus and enzyme cocktail, and sperm concentrations measured at liquefaction time.

The concentration of spermatozoa measured in liquefied samples was compared to that in the initial samples (calculated values). An overall correlation coefficient of 0.999 was observed for concentrations ranging from 10 000 to 5×10^6 spermatozoa/ml (data not shown). For more detailed analysis, particularly in the low concentration range (which may be critical for PCT evaluation), sperm recovery was expressed as a percentage of sperm concentration in liquefied samples with respect to concentrations measured in control samples at time $t = 0$. Figure 5 shows the percentage of sperm recovered after liquefaction as a function of sperm concentration. We have obtained accurate measurements of sperm concentrations at 0.5×10^6 spermatozoa/ml and above, with an average recovery of $97 \pm 9\%$ (mean \pm SD, $n = 38$). Below 0.5×10^6 spermatozoa/ml, concentrations could not be accurately measured in Makler chambers, since fewer than five cells were counted per chamber. Between 0.1×10^6 and 0.4×10^6 spermatozoa/ml, detection of spermatozoa was reliable, but the quantitative measurements lacked precision ($103\% \pm 31\%$, $n = 22$). At $<0.1 \times 10^6$ spermatozoa/ml, detection of spermatozoa was

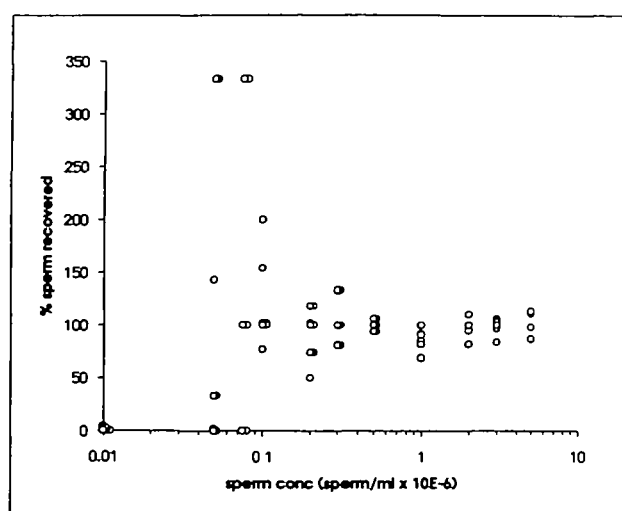


Figure 5. Sensitivity of sperm counts in liquefied cervical mucus. Spermatozoa at various concentrations were incubated with donor cervical mucus and the enzyme cocktail, and the number of sperm cells recovered was measured at liquefaction time. Sperm counts were normalized to control samples at $t = 0$ where cervical mucus was replaced by buffer, and plotted against sperm concentrations. For each sperm concentration tested, six to eight different samples were analysed. Sperm concentration measurements were found to be accurate at concentrations $\geq 0.5 \times 10^6$ spermatozoa/ml (recovery = $97 \pm 9\%$, mean \pm SD, $n = 38$). Sperm cells were always found in samples containing $0.1\text{--}0.4 \times 10^6$ sperm/ml, but the concentrations measured were inaccurate ($103 \pm 31\%$, $n = 22$). Detection of spermatozoa at concentrations $< 0.1 \times 10^6$ spermatozoa/ml was not reliable and actual sperm concentrations could not be measured.

not reliable, and concentrations could not be measured. These threshold concentrations were validated by one-way analysis of variance (Levene's test) which showed significant differences in variability of the sperm concentration only when values $< 0.5 \times 10^6$ were included in the analysis. Therefore, the limit of sensitivity for accurate sperm concentration determination was 0.5×10^6 spermatozoa/ml. Qualitative sperm detection was possible at concentrations ranging from 0.1 to 0.4×10^6 spermatozoa/ml. These ranges of sensitivity corresponded to the sensitivity of measurement of the Makler cell itself, which appeared to be the main limiting factor in the system. Thus, our protocol for enzymatic liquefaction of cervical mucus did not seem to interfere with subsequent sperm counts and concentration measurements.

Discussion

Repeated criticisms have challenged the validity of the PCT as being poorly related to fertility (Hartman, 1957; Griffith and Grimes, 1990; Markham, 1991) and this controversy is partly due to technical problems that are responsible for variability in the results. The measurement of sperm concentration in PCT samples has been proposed to improve the quantitative evaluation of PCT (Campana *et al.*, 1991; Doody and Good, 1993; de Agostini *et al.*, 1995). In a recent report, Doody and Good (1993) have adapted a method originally proposed by Drobnis *et al.* (1988) using calibrated beads to maintain a constant distance between slide and coverslip when

examining cervical mucus samples, thereby allowing sperm concentration measurements in PCT. The precision of such measurements is, however, limited by the non-homogeneous distribution of spermatozoa in PCT mucus. To solve this problem we proposed to measure sperm concentration in PCT after enzymatic liquefaction of the cervical mucus (de Agostini *et al.*, 1996). However, the reliability of these measurements could not be assessed using PCT samples where the absolute number of sperm present is unknown. We have addressed this issue in the present study using as a model, donor cervical mucus to which known amounts of spermatozoa were added before liquefaction. The results presented demonstrate that sperm concentrations can be measured with adequate accuracy and sensitivity in enzymatically liquefied cervical mucus and thus validate the use of enzymatic liquefaction to measure sperm concentrations in PCT.

The donor cervical mucus used in this study was collected from gonadotrophin-stimulated patients. This condition favours the production of abundant high quality pre-ovulatory cervical mucus (Eggert-Kruse *et al.*, 1989), which mimics PCT samples with high cervical mucus scores. Interpretation of the PCT in terms of sperm penetration requires the cervical mucus score to be adequate (i.e. > 10), since low score mucus constitutes a hostile environment for spermatozoa (WHO, 1992). Therefore gonadotrophin-treated donor cervical mucus is a good model to evaluate the effects of enzyme treatments on sperm concentrations in PCT. The use of donor cervical mucus made it possible to compare the effects of different treatments on aliquots of the same mucus, as well as to measure sperm recovery by adding known amounts of spermatozoa to cervical mucus samples; such comparisons are not possible in PCT.

We have tested the ability of bromelin, either alone or in conjunction with α -amylase and hyaluronidase (the enzyme cocktail), to liquefy cervical mucus. Bromelin has been used to liquefy cervical mucus to detect antibodies, leukocytes and sperm cells (Ingerslev and Poulsen, 1980; Campana *et al.*, 1991; Thompson *et al.*, 1991; Shulman and Hu, 1992). In our hands, however, bromelin liquefaction of PCT samples yielded widespread distribution of liquefaction times (de Agostini *et al.*, 1996). Therefore we used donor cervical mucus samples, each split into two aliquots, to compare the efficiency of bromelin alone and of the enzyme cocktail in achieving reliable rapid mucus liquefaction. The aliquots incubated with the enzyme cocktail were liquefied in significantly shorter times than those incubated with bromelin alone. Indeed, the enzyme cocktail allowed liquefaction of most of the mucus samples within 60 min of incubation, which proved to be more favourable for standard laboratory procedure. The fact that shorter liquefaction times were obtained using the enzyme cocktail as opposed to bromelin in aliquots of the same donor mucus excludes the possibility that such differences could be due to differences in mucus score. However, in some of the donor mucus samples, liquefaction was largely delayed when using bromelin alone, indicating the existence of subtle individual variations that are not reflected in the mucus score. The fact that these differences were minimized in the presence of glycosidases suggests that individual mucus samples may differ with regard to their carbohydrate composition. The

composition of cervical mucus is known to vary during the cycle, and mid-cycle cervical mucus has been shown to contain decreased total amounts of proteins and maximal amounts of mucin-type high molecular size glycoproteins (Morales *et al.*, 1993).

In parallel, we have examined the effects of enzyme treatments on spermatozoa themselves. Treatment with bromelain or with the enzyme cocktail affected sperm motility (data not shown) and therefore motility measurements in PCT were done before liquefaction of cervical mucus. Sperm cells were found to be sensitive to the enzymes used for cervical mucus liquefaction, resulting in a moderate decrease in sperm counts over time. This effect was maximal in control experiments where sperm cells were incubated with enzymes in the absence of mucus. In the presence of cervical mucus, sperm counts were markedly decreased only when further incubated with enzymes after liquefaction completion. This suggests that cervical mucus constituents protected sperm cells from lysis by providing alternate substrates for the enzymes. In the light of these observations, it is tempting to speculate that in PCT conditions, where spermatozoa are embedded within the cervical mucus, the sperm cells would be better protected against lysis than in our present experimental conditions. Moreover, the fact that bromelain alone does not affect sperm counts even after prolonged incubation implies that glycosidases present in the enzyme cocktail are involved in sperm lysis. It seems unlikely that glycosidases alone would lyse spermatozoa, since these cells are physiologically exposed to α -amylase, which is present in cervical mucus (Vermeiden *et al.*, 1989), and to endogenous hyaluronidase, which is released during the acrosome reaction (Sidhu and Guraya, 1989). Sperm surfaces are coated by numerous macromolecules including glycoproteins and polylactosaminoglycans (Sidhu and Guraya, 1989; Zaneveld *et al.*, 1991) that could protect sperm membrane proteins from proteolytic attack. Glycosidases could gradually degrade this protective glycosidic coat and unmask membrane proteins, rendering them susceptible to proteolytic cleavage and ultimately leading to sperm lysis. The demonstration of such a mechanism would require further experimentation.

In our experimental conditions, however, we have demonstrated that in the presence of cervical mucus, enzymatic sperm lysis remains marginal and that accurate measurements of sperm concentrations can be made in solubilized cervical mucus at liquefaction time.

The sensitivity limit for quantitative sperm concentration measurement in cervical mucus liquefied with the enzyme cocktail is 0.5×10^6 spermatozoa/ml, although at lower concentrations, between 0.1 and 0.4×10^6 spermatozoa/ml, reliable qualitative detection of sperm cells is also achieved. The limit of sensitivity for sperm concentration measurement corresponds to the lower limit of confidence of the Makler counting chamber used in this study, which demonstrates that our procedure for mucus liquefaction does not decrease the sensitivity of sperm counts as compared to direct measurements of spermatozoa in semen. If necessary, the sensitivity of sperm concentration measurement could be improved by increasing the volume of sample examined. This could be done by counting multiple

aliquots of the same sample (for example 10) or by using a different counting chamber such as a haemocytometer with a counting volume ($0.1 \mu\text{l}$) 10-fold bigger than a Makler counting chamber.

In PCT, sperm concentrations have to be multiplied by a factor of two to take into account the 1:1 dilution of the mucus sample with the enzyme cocktail (de Agostini *et al.*, 1996). Thus, accurate quantitative measurement of sperm concentrations can be obtained in PCT cervical mucus liquefied with the enzyme cocktail with a lower sensitivity limit of 1×10^6 spermatozoa/ml cervical mucus. Accordingly, reliable qualitative detection of sperm cells in liquefied PCT is achieved between 0.2 and 0.9×10^6 spermatozoa/ml cervical mucus.

We have examined the data reported in the accompanying paper on PCT (de Agostini *et al.*, 1996) in the light of these sensitivity limits. In a series of 36 PCT samples 27 had sperm concentrations $>10^6$ spermatozoa/ml and in these cases the estimate of total sperm content was accurate. The remaining nine samples (25%) had sperm concentrations between 0.2 and 1×10^6 spermatozoa/ml and these samples all had a total sperm content $<10^6$ spermatozoa. For these latter cases, the estimate of their total sperm content was qualitative, due to the limited accuracy of measurements at low concentrations. We also examined the accuracy of sperm concentrations measured in PCT samples scoring below the limit of normality according to WHO (1992) guidelines (10 spermatozoa/hpf). Among 42 PCT samples with <10 spermatozoa/hpf observed before liquefaction, sperm concentrations were accurately measured in 18 samples (43%) containing $>1 \times 10^6$ spermatozoa/ml; sperm cells were qualitatively detected in 14 samples (33%) with measured sperm concentrations included in the range between 0.2 and 0.9×10^6 spermatozoa/ml; and spermatozoa could not be detected in 10 samples (24%) with a sperm concentration $<0.2 \times 10^6$ sperm/ml.

Attempts have been made to improve the accuracy of analyses of sperm-mucus interactions in relation to fertility using in-vitro penetration assays. Recently, the distance of migration of the vanguard spermatozoa in a mucus column has been used to assess the effects of anti-oestrogens, used to induce ovulation, on the ability of sperm cells to penetrate cervical mucus (Acharya *et al.*, 1993). In another study, human donor cervical mucus and bovine cervical mucus have been compared for their ability to predict PCT results and to discriminate between normal and abnormal semen using the same technique (Sharara *et al.*, 1994). The results from normal and abnormal semen were found to be widely overlapping, and did not always predict PCT outcome correctly. The authors concluded by questioning the validity of the in-vitro penetration assay as a substitute for PCT. Such studies could be extended by measuring the concentration of sperm that entered cervical mucus, or could be conducted on PCT to measure directly the numbers of spermatozoa present in cervical mucus in relation to treatment or sperm quality. The in-vitro sperm penetration assay has advantages over the PCT, since it can be conducted in better controlled conditions. On the other hand, PCT samples are closer to the physiological condition of the patient and thus more clinically relevant. Therefore, improvement of the quantitative evaluation of PCT samples, by measuring sperm

concentrations in liquefied mucus, is highly desirable for adequate evaluation of sperm–cervical mucus interactions.

In conclusion, we have quantitatively validated a new method for cervical mucus liquefaction that will allow accurate measurements of sperm concentrations in cervical mucus from PCT or from sperm–cervical mucus penetration assays. We have demonstrated that a combination of protease and glycosidases efficiently dissolves cervical mucus and allows quantitative recovery of spermatozoa in the liquefied samples. This method is reproducible and allows the concentration of sperm cells in cervical mucus to be determined and the total number of spermatozoa present in cervical mucus at the time of PCT to be estimated. The availability of quantitative data should help to improve the interpretation of PCT results in relation to fertility predictions. In addition, such quantitative data may improve our knowledge of the physiology of sperm–mucus interactions in terms of estimating the number of sperm cells reaching the upper genital tract.

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References

- Acharya, U., Irvine, D.S., Hamilton, M.P. and Templeton, A.A. (1993) The effect of three anti-oestrogen drugs on cervical mucus quality and in-vitro sperm–cervical mucus interaction in ovulatory women. *Hum. Reprod.*, **8**, 437–441.
- Biggers, J.D., Whitten, W.K. and Whittingham, D.G. (1971) The culture of mouse embryos *in vitro*. In Daniel, J.C. (ed.), *Methods in Mammalian Embryology*. W.H. Freeman and Co., San Francisco, pp. 86–116.
- Campana, A., Ferla, E., Marossi, L., Radici, E., Stalberg, A. and Balerna, M. (1991) Eine neue Methode zur Beurteilung des Postkoitaltests. *Fertilität*, **7**, 181–184.
- de Agostini, A.I., Tawfik, E. and Campana, A. (1996) Quantitative post-coital test: sperm counts in cervical mucus after enzymatic liquefaction. *Hum. Reprod.*, **11**, 311–317.
- Doody, M.C. and Good, M.C. (1993) The post-coital test: a quantitative method. *J.Androl.*, **14**, 149–154.
- Drobnis, E.Z., Yudin, A.I., Cherr, G.N. and Katz, D.F. (1988) Hamster sperm penetration of the zona pellucida: kinematic analysis and mechanical implications. *Dev. Biol.*, **130**, 311–323.
- Eggert-Kruse, W., Leinhos, G., Gerhard, I., Tilgen, W. and Runnebaum, B. (1989) Prognostic value of *in vitro* sperm penetration into hormonally standardized human cervical mucus. *Fertil. Steril.*, **51**, 317–323.
- Griffith, C.S. and Grimes, D. (1990) The validity of the post-coital test. *Am. J. Obstet. Gynecol.*, **162**, 615–620.
- Hartman, C.G. (1957) How do sperm get in the uterus? *Fertil. Steril.*, **8**, 403–427.
- Ingerslev, H.J. and Poulsen, F. (1980) Bromelin for liquefaction of cervical mucus in sperm antibody testing: its effect on spermagglutinating immunoglobulin G. *Fertil. Steril.*, **33**, 61–63.
- Kremer, J. and Jager, S. (1988) Sperm–cervical mucus interaction, in particular in the presence of antispermatozoal antibodies. *Hum. Reprod.*, **3**, 69–73.
- Markham, S. (1991) Cervico-utero-tubal factors in infertility. *Curr. Opin. Obstet. Gynecol.*, **3**, 191–196.
- Morales, P., Roco, M. and Vigil, P. (1993) Human cervical mucus: relationship between biochemical characteristics and ability to allow migration of spermatozoa. *Hum. Reprod.*, **8**, 78–83.
- Sakkas, D., Jaquenoud, N., Leppens, G. and Campana, A. (1994) Comparison of results after *in vitro* fertilized human embryos are cultured in routine medium and in coculture on Vero cells: a randomized study. *Fertil. Steril.*, **61**, 521–525.
- Sharara, F.I., Beatse, S.N., Bailey, S.A., Neal, G.S., Coddington, C.C. and Scott, R.T.J. (1994) Characterization of Tru-Trax in-vitro penetration testing of cervical mucus. *Hum. Reprod.*, **9**, 2027–2031.
- Shulman, S. and Hu, C. (1992) A study of the detection of sperm antibody in cervical mucus with a modified immunobead method. *Fertil. Steril.*, **58**, 387–391.
- Sidhu, K.S. and Guraya, S. (1989) Cellular and molecular biology of capacitation and acrosome reaction in mammalian spermatozoa. *Int. Rev. Cytol.*, **118**, 231–280.
- Thompson, L.A., Tomlinson, M.J., Barratt, C.L., Bolton, A.E. and Cooke, I.D. (1991) Positive immunoselection – a method of isolating leukocytes from leukocytic reacted human cervical mucus samples. *Am. J. Reprod. Immunol.*, **26**, 58–61.
- Vermeiden, J.P.W., Bernardus, R.E., ten Brug, C.S., Statema-Lohmeijer, C.H., Willemsen-Brugma, A.M. and Schoemaker, J. (1989) Pregnancy rate is significantly higher in *in vitro* fertilization procedure with spermatozoa isolated from nonliquefying semen in which liquefaction is induced by alpha-amylase. *Fertil. Steril.*, **51**, 149–152.
- WHO (1987) *WHO Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 2nd edn. Cambridge University Press, Cambridge, pp. 1–67.
- WHO (1992) *WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction*, 3rd edn. Cambridge University Press, Cambridge, pp. 1–107.
- Zaneveld, L.J., De Jonge, C.J., Anderson, R.A. and Mack, S.R. (1991) Human sperm capacitation and the acrosome reaction. *Hum. Reprod.*, **6**, 1265–1274.

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